

# Apoptosis

## Basic Mechanisms and Implications for Cardiovascular Disease

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### 1. Introduction

Since Kerr et al<sup>1</sup> in 1972 coined the term "apoptosis" for a morphologically distinct mode of cell death, this concept of cell suicide has gained increasing interest in cytology and pathology. In terms of tissue kinetics, apoptosis may be considered a mechanism that counterbalances the effect of cell proliferation by mitotic division. In fact, deregulated apoptosis has been implicated as a fundamental pathogenetic mechanism in a variety of human diseases. Excessive apoptotic cell death may cause organ atrophy and organ failure, as suggested for neurodegenerative diseases and viral hepatitis. On the other hand, inefficient elimination of malignant, autoreactive, infected, or redundant cells may lead to the development of neoplasia, autoimmunity, viral persistence, and congenital malformations. Further interest in apoptosis has arisen from the recent elucidation of effector and regulatory mechanisms with the aid of molecular biology, genetics of lower organisms, and genetic modification of the mouse. However, only recently, compelling evidence has accumulated indicating that apoptotic cell death may also play a critical role in a variety of cardiovascular diseases, including myocardial infarction, heart failure, and atherosclerosis.

Apoptosis can be differentiated from other forms of cell death that occur in response to toxins, physical stimuli, and ischemia. Although not widely used, this form of cell death was termed "accidental cell death" in the pathology literature.<sup>2</sup> In contrast to apoptosis, accidental cell death does not involve suicide mechanisms and is not energy dependent. In the case of accidental cell death induced by ischemia, depletion of intracellular ATP stores, swelling, and disruption of the cell membrane, leading to liberation of cytoplasmic contents into the extracellular space, are prominent features (Figure 1). This specific form of accidental cell death is also referred to as "oncosis." The term "necrosis," often used to describe cell death other than apoptotic cell death, is imprecise, because it actually refers to irreversible cell and/or tissue alterations visible on microscopy irrespective of whether cell death is apoptotic or accidental.<sup>2</sup> For the scope of clarity and simplicity, we will refer to cell death characterized by caspase activation and caspase-mediated protein cleavage and internucleosomal DNA fragmentation as apoptotic (Figure 1).

Other forms of cell death will be collectively summarized under the term "nonapoptotic cell death."

Although some of the features of apoptosis have previously been summarized in several recent reviews,<sup>3-8</sup> research in apoptosis is progressing at a tremendous pace, and interesting new insights into the mechanisms of apoptosis have been gained since then. Because most of these findings have been made and published in the fields of immunology and oncology, it is the scope of this review both to provide an overview summarizing the important literature involving molecular mechanisms of apoptosis in general and to summarize current specific knowledge about apoptosis in cardiovascular disease. The morphological alterations associated with apoptosis will be addressed in the second section of this article. The third and fourth sections will review structure and function of caspases and mechanisms leading to their activation. On the basis of current evidence, mechanisms that depend on the activation of apoptosis-inducing cell surface receptors and on the mitochondrial release of proapoptotic factors will be addressed separately. Because apoptosis affects the basic function of a cell, namely cell viability, several mechanisms that regulate its initiation have evolved. These include mechanisms specific to apoptosis regulation (eg, bcl-2 family proteins) and signal transduction pathways involved in other cellular functions (eg, SAPKs). These are discussed in detail in sections 5 and 6, respectively. In addition, several reports suggest that apoptosis provides a safeguard against deregulated cell proliferation. Because this may be of pathophysiological significance in cardiovascular disease, this aspect was also included in the present review (section 7). Section 8 will give an overview of current evidence for apoptosis in cardiac development, heart failure, ischemic heart disease, and atherosclerosis. The final section (section 9) will summarize unanswered questions and the authors' perspective on future directions.

### 2. Morphology and Identification of the Apoptotic Cell

#### Morphology

Apoptosis is a distinct form of cell death that displays characteristic alterations in cell morphology and cell fate.<sup>2</sup> Chromatin condensation and margination that result in a "half-moon" or "horseshoe" appearance of the nucleus are

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**Selected Abbreviations and Acronyms**

AIF	= apoptosis-inducing factor
ANF	= atrial natriuretic factor
apaf	= apoptotic protease-activating factor
ARC	= apoptosis repressor with caspase recruitment domain
BH	= bcl-2 homology
CASH	= caspase homologue
CD	= cluster of differentiation
DED	= death effector domain
DR	= death receptor
ERK	= extracellular signal-regulated kinase
FADD	= Fas-associated death domain protein
FasL	= Fas ligand
FLAME-1	= FADD-like antiapoptotic molecule
FLICE	= FADD-homologous ICE/Ced-3-like protease
FLIP	= FLICE-inhibitory proteins
I $\kappa$ B	= inhibitor of NF $\kappa$ B
I-FLICE	= inhibitor of FLICE
IAP	= inhibitor of apoptosis protein
ICE	= interleukin-1 $\beta$ -converting enzyme
IGF	= insulin-like growth factor
JNK	= c-Jun N-terminal kinase
MAPK	= mitogen-activated protein kinase
MEK	= MAPK/ERK kinase
MEKK	= MEK kinase
NF $\kappa$ B	= nuclear factor $\kappa$ B
NGF	= nerve growth factor
PI-3 kinase	= phosphatidylinositol-3 kinase
PKB, PKC	= protein kinase B and C
RAIDD	= RIP-associated ICH-1/Ced-3-homologous death domain protein
RIP	= receptor-interaction protein
SAPK	= stress-activated protein kinase
SEK	= SAPK/ERK kinase
TdT	= terminal deoxynucleotidyltransferase
TNF	= tumor necrosis factor
TNFR	= TNF receptor
TRADD	= TNFR-associated death domain protein
TRAF	= TNFR-associated factor
TRAIL	= TNF-related apoptosis-inducing ligand
TUNEL	= TdT-mediated dUTP nick end-labeling
zVAD.fmk	= benzyloxycarbonyl-valine-alanine-aspartate fluoromethylketone

typical features of apoptotic cell death.<sup>2</sup> Although morphological alterations of mitochondria may be subtle, mitochondrial function was reported to be irreversibly impaired early in apoptosis.<sup>9,10</sup> In addition, cytoskeletal alterations and membrane budding can be observed.<sup>2,11</sup> In later stages of apoptosis, nuclear fragmentation becomes evident (karyorrhexis), the cytoplasm condenses progressively, and one or more apoptotic bodies are formed from each dying cell. The cell remnants are taken up by phagocytic cells of the macrophage/monocyte lineage. Interestingly, apoptotic bodies may also be engulfed by cells not specialized in phagocytosis (eg, vascular smooth muscle cells).<sup>12</sup> Nuclear condensation, nuclear fragmentation, and sequestration of cell fragments have been documented in cardiomyocytes *in situ*.<sup>13,14</sup> Similar features have been observed in apoptotic smooth muscle cells of atherosclerotic lesions by light and electron microscopy, indicating that myocardial and vascular cells show at least some of the cytological features described in other cell types.<sup>15-18</sup>

**Glossary****Bcl-2 Family Proteins**

This class of proteins shows homology to the *C. elegans* protein ced-9. The first member of this protein family was named B-cell lymphoma 2 gene (bcl-2), because it proved to induce lymphomas in humans when activated by chromosome translocation. In mammalian species, both proapoptotic and antiapoptotic members of this protein family have been characterized. Bcl-2 family proteins are localized to the outer mitochondrial and nuclear membranes and to the membrane of the endoplasmic reticulum.

**Caspases**

Caspases are intracellular apoptosis-associated proteases that cleave substrate proteins behind aspartate residues. They characteristically contain a cysteine residue in the catalytic center.

**ced-3, ced-4, and ced-9**

Programmed cell death of a defined set of cells is an inherent feature in the development of the nematode *C. elegans*. Genetic analysis of mutants with disturbances in developmental cell death identified a group of cell death genes (ced). Apart from genes involved in the removal of dead cells, the genes ced-3, ced-4, and ced-9 proved to be instrumental in the execution and regulation of programmed cell death. Characterization of these genes was seminal in the understanding of genes involved in the apoptosis of mammalian cells, such as caspases (ced-3), apaf-1 (ced-4), and bcl-2-related proteins (ced-9).

**Death Receptors**

Death receptors are a class of cell membrane receptors belonging to the larger group of TNF receptors. Death receptors are characterized by an intracytoplasmic death domain of ~80 amino acid residues. Members of this group comprise Fas, TNFR1, DR3, DR4, and DR5. The cognate ligands are Fas ligand (FasL), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Apo-3L, and TNF-related apoptosis-inducing ligand (TRAIL) for Fas, TNFR1, DR3, and DR4/5, respectively.

**Fas**

Fas, the prototypical death receptor, mediates apoptotic cell death after stimulation by Fas ligand. Receptor activation involves the recruitment of adaptor proteins to the cell membrane and subsequent caspase activation.

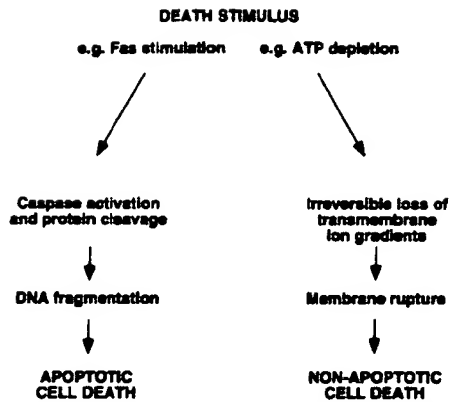
**Inhibitor of Apoptosis Proteins**

Inhibitor of apoptosis proteins (IAPs) are a class of antiapoptotic proteins that were initially isolated in baculovirus, a virus infecting insect cells. Mammalian homologues are believed to inhibit apoptosis by direct caspase inhibition and by mediating survival signals after TNFR stimulation.

**p53**

p53 is a transcriptional transactivator protein that is involved in cell cycle control and DNA repair. p53 has been implicated in apoptosis induced by genotoxic agents and deregulated cell cycle control.

Attachment of apoptotic cell remnants to the phagocytosing cell appears to be mediated by various pathways. Interaction of phosphatidylserine on the cell surface of the apoptotic cell with phosphatidylserine receptors on the phagocytosing cells constitutes one major mechanism.<sup>12,19</sup> In most cell membranes, an asymmetry of phospholipids is



**Figure 1.** Classification of cell death based on the mechanisms believed to cause cell death. Nonapoptotic cell death associated with cell swelling is also referred to as oncosis. Fas indicates the death receptor Fas on the cell surface that can mediate ligand-induced cell apoptosis on stimulation.

maintained, with phosphatidylserine and phosphatidylethanolamine being confined to the inner leaflet of the cell membrane.<sup>20</sup> Possibly, through activation of a lipid scramblase or inhibition of the enzyme aminophospholipid translocase, this asymmetry of membrane composition is lost during apoptosis, leading to exposure of phosphatidylserine on the cell surface that can be recognized by specific receptors.<sup>20</sup> An alternative mechanism involves the vitronectin receptor, a protein of the integrin family present on fibroblasts and macrophages.<sup>19,21,22</sup> However, other mechanisms, such as lectin-mediated binding of sugar residues or CD14- and CD36-mediated binding, have been suggested.<sup>23,23a</sup> In the central core of atherosclerotic lesions, lipid loading and apoptosis of macrophages may interfere with efficient removal of apoptotic cell remnants, leading to accumulation of cell debris in the central core.<sup>24</sup>

### Assays for Apoptosis

How can apoptosis be verified in tissue samples? The most convincing proof for apoptosis in tissue sections is the detection of apoptotic bodies containing nuclei with characteristic features of apoptosis. These are best visualized by electron microscopy. However, because apoptotic bodies are phagocytosed within hours after cell death, apoptotic cell remnants may be difficult to verify whenever apoptosis affects only a limited number of cells or occurs only transiently. Nuclear staining with the fluorescent dye bisbenzimidazole (Hoechst 22358) allows for the visualization of nuclear condensation and fragmentation in both cell culture and tissue sections.

The most commonly used techniques to detect apoptosis are based on the fact that during apoptosis the genomic DNA is cleaved within internucleosomal DNA segments by an endonuclease selectively activated during apoptosis.<sup>25,26</sup> Internucleosomal DNA fragmentation appears to be preceded by the formation of larger DNA fragments with lengths of 50 and 300 kb.<sup>27,28</sup> In some cells, degradation to small internucleosomal fragments may not take place at all.<sup>28</sup> Separation of cellular DNA on agarose gels shows a characteristic ladder-like pattern of fragments with multiples of 200 bp in length.

The disadvantage of this technique is that it is not possible to assign the apoptotic process to a specific cell type whenever tissue samples containing different cell populations are analyzed.

Therefore, other techniques that allow for histochemical analysis have been developed. In the TUNEL (TdT-mediated dUTP nick end-labeling) technique, dUMP is attached to the 3' end of genomic DNA by TdT (terminal deoxynucleotidyl-transferase). Positive cells are visualized by fluorescent dyes conjugated to dUTP.<sup>29</sup> An alternative technique, *in situ* end-labeling (ISEL), uses the Klenow fragment of *Escherichia coli* DNA polymerase I that recognizes only 3' recessed ends, whereas TdT processes any type of free 3' ends.<sup>30</sup> Comparative analysis suggests a higher sensitivity for the TUNEL technique in the detection of apoptosis.<sup>31</sup> However, a cautious note needs to be added, because exposure of 3' DNA ends is not a unique feature of apoptosis and may occur during DNA repair and nonspecific DNA damage, potentially limiting the specificity of this technique.<sup>32,33</sup> Sensitivity for the detection of DNA fragmentation in individual cultured cells may be increased by the "comet assay."<sup>34,35</sup> In this assay, cells are lysed in agarose and subjected to gel electrophoresis. Fragmented DNA migrates out of the nucleus and forms a characteristic comet tail. Although this technique displays a high sensitivity for DNA breaks, it does not provide definite proof of the internucleosomal DNA cleavage characteristic of apoptosis.<sup>36</sup>

Because the enzymatic fragmentation of the DNA is a late event in apoptosis, there is an ongoing search for markers that allow for the detection of cells that are still in the early phases of apoptosis. Current evidence suggests that the binding of annexin V to phosphatidylserine exposed on the outer leaflet of the cell membrane may constitute an advance in this direction.<sup>23</sup> In addition, specific cleavage of cellular target proteins, such as poly(ADP)-ribosylating protein, is considered to be a hallmark of apoptosis.<sup>6,8,36</sup> Detection of protein fragments by Western blotting has been used to verify apoptotic cell death in cell culture models of apoptosis.

### 3. Caspases as the Effector Machinery of Apoptosis

A key phenomenon of apoptotic cell death is the activation of a unique class of aspartate-specific proteases. Until now, at least 10 members have been identified in this subclass of proteases (Table 1).<sup>6</sup> In order to simplify the confusing terminology of the known aspartate-specific proteases, a new nomenclature has been adopted, classifying all aspartate-specific proteases under the term caspases.<sup>37</sup> Crystal structure analysis of the prototypical caspase ICE (caspase-1) revealed that the serine residue common to many other proteases is replaced by a cysteine residue within a highly conserved pentameric sequence in the catalytic center.<sup>38,39</sup> All caspases are composed of a prodomain and an enzymatic region (Figure 2). Heterogeneity among the proteases exists regarding the structure of the prodomain, suggesting that this region may define important functional differences between caspases. Caspases-1, -2, -4, -5, -8, -9, and -10, like their *Caenorhabditis elegans* homologue ced-3, contain a long prodomain of ~15 to 25 kDa compared with <5 kDa in

TABLE 1. Caspases

New Nomenclature	Previous Nomenclature	Prodomain	MW, kDa
Caspase-1	ICE	Long	45
Caspase-2	ICH-1	Long	48
Caspase-3	CPP32, Yama, apopain	Short	32
Caspase-4	TX, ICH-2, ICErel-II	Long	43
Caspase-5	TY, ICErel-III	Long	48
Caspase-6	Mch2	Short	34
Caspase-7	Mch3, ICE-LAP3, CMH-1	Short	35
Caspase-8	FLICE, Mach, Mch5	Long	55
Caspase-9	ICE-LAP6, Mch6	Long	46
Caspase-10	Mch4	Long	55

MW indicates molecular mass. Refer to References 6 and 37.

caspases-3, -6, and -7 (Figure 2 and Table 1). For activation, the caspase proform has to be cleaved into a large subunit and a small subunit within the enzymatic domain that finally reassociate to form a complex comprising 2 small and 2 large subunits. The prodomain is not necessary for the proteolytic activity once the caspase is activated. Interestingly, all activating cleavages occur behind an aspartate residue. Because this cleavage site is a unique characteristic of caspases (with the serine protease granzyme B being the only exception), activation can occur only through autoactivation or cleavage by another caspase or granzyme B.

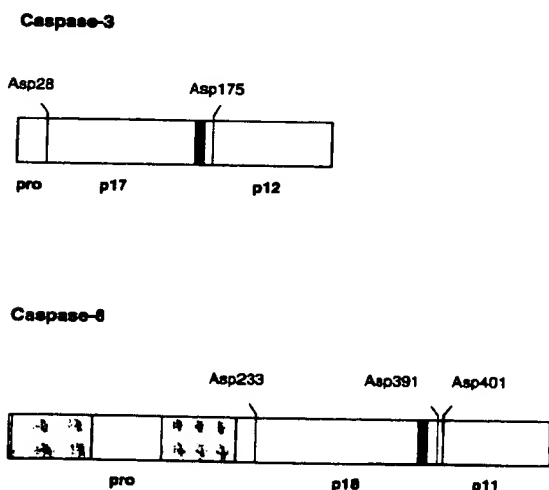
In contrast to the nematode *C. elegans*, where only one caspase, ced-3, has been identified, we face the dilemma of defining the specific functional role of several different members of this protease family in the mammalian system.<sup>6,40</sup> Do they function in a sequential activation cascade as in the coagulation system, or do they act in parallel, forming at least

partially redundant pathways? Surprisingly, phenotypic analysis of mice with a deficiency of the prototypical mammalian ced-3 homologue ICE (caspase-1) exhibited normal intrauterine and postnatal development.<sup>41</sup> Although caspase-1<sup>-/-</sup> thymocytes showed impaired Fas-induced apoptosis in vitro, a phenotype similar to mice deficient in the apoptosis-mediating receptor Fas or its ligand with lymphoid hyperplasia and autoimmunity could not be observed.<sup>41</sup> In addition, sensitivity to radiation and glucocorticoids was not reduced, suggesting that caspase-1 does not play a major role within the apoptotic machinery or that it can efficiently be replaced by other caspases. In contrast, mice deficient in caspase-3 exhibited a striking phenotype of multiple neuronal hyperplasia in the brain consistent with inefficient cell elimination during fetal development.<sup>42</sup> Changes in brain histology became obvious from gestational day 12 on and resulted in markedly reduced prenatal and postnatal viability. Intriguingly, no abnormalities were found in other tissues, including the heart and the immune system, where apoptosis is known to play a major role in cell maturation. A possible explanation is that in those tissues redundant caspase activities are expressed that compensate for the loss of caspase-3 function.

Apart from differences in prodomain length, caspases can be grouped according to the sequence requirement surrounding the target aspartate residue. Caspases-6, -8, and -9 preferentially cleave protein substrates at (valine/leucine)-glutamate-(threonine/histidine)-aspartate (V/L-E-T/H-D), whereas caspases-3 and -7 show a high selectivity for a peptide motif consisting of aspartate-glutamate-valine-aspartate (DEVD).<sup>43</sup> The optimal target sequence for caspase-1 and the related caspases-4 and -5 is believed to be tyrosine-valine-alanine-aspartate (YVAD) or the tetrapeptide (tryptophan/leucine)-glutamate-histidine-aspartate (W/L-EHD).<sup>43</sup>

Enari et al<sup>44</sup> showed that in apoptosis induced by stimulation of the death receptor Fas, caspases that are competitively inhibited by a YVAD oligopeptide are upstream from caspases that are inhibited by DEVD peptides (eg, caspases-3 and -7), suggesting the sequential activation of caspase subgroups. Recruitment of caspases-2 and -8 through their prodomains to the Fas receptor complex supports the notion that these proteases are activated early through a receptor-dependent mechanism.<sup>45-49</sup> This may also be true for caspase-10, whose prodomain shows a high homology to the prodomain of caspase-8.<sup>50</sup>

Target proteins for caspases comprise a plethora of different proteins, including nuclear proteins, proteins involved in signal transduction, and cytoskeletal targets (Table 2).<sup>6,8,51-53</sup> Most of these protein substrates appear to be cleaved by caspases-3 and -7. However, lamin is selectively cleaved by caspase-6.<sup>54,55</sup> Although many of the target proteins defined to date have a nuclear localization, apoptotic cell death does not depend on the presence of a cell nucleus, as the characteristic cytoplasmic features of apoptosis can be observed in anucleate cytoplasts.<sup>56</sup> Therefore, proteolytic cleavage of nuclear proteins may be important in eliciting the nuclear features of apoptosis, like chromatin margination induced by lamin B cleavage, but does not constitute a critical event for the apoptotic death of the entire cell.<sup>57,58</sup> Interestingly, internucleosomal DNA fragmentation requires the prior cleavage of



**Figure 2.** Structural characteristics of caspase-3 and -8. The black segment within the large catalytic subunit indicates the catalytic center of the caspase. The shaded subregions within the prodomain of caspase-8 represent the death effector domains required for the recruitment of caspase-8 to the activated death receptor complex. Asp indicates the cleavage sites for activation of the caspases. Pro indicates the prodomain; p12/p17 and p11/p18, the enzymatic subunits of caspase-3 and -8, respectively.

**TABLE 2. Downstream Targets of Caspases**

Nuclear proteins
Lamin
Rb protein
DNA-dependent protein kinase
70-kDa subunit of U1 small nuclear ribonucleoprotein
Poly (ADP)-ribosylating protein (PARP)
Mdm2
Regulatory proteins
MAPK/ERK kinase kinase 1 (MEKK1)
Protein kinase C $\delta$
G4-GDI GDP dissociation inhibitor
Sterol regulatory element binding protein
DNA fragmentation factor/inhibitor of caspase-activated DNase
Cytoskeletal proteins
Fodrin
Gelsolin
Actin
Gas2

Refer to References 6, 8, and 51 to 53.

a cytoplasmic inhibitor of the apoptosis-specific endonuclease (inhibitor of caspase-activated DNase, DNA fragmentation factor).<sup>26,53,57</sup> Only after cleavage of the inhibitor can the endonuclease translocate to the nucleus and degrade genomic DNA.<sup>26,53</sup> For most of the downstream targets, the overall contribution to the final cell fate remains to be determined.

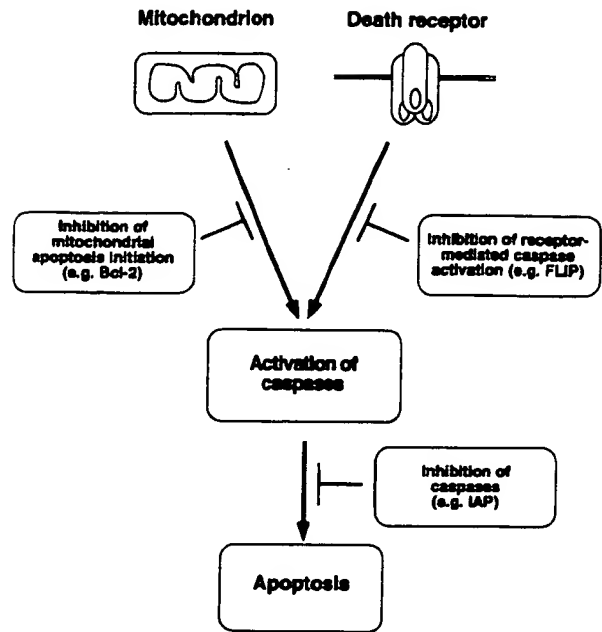
In summary, caspases can be grouped into an upstream and a downstream subgroup. Upstream caspases are characterized by long prodomains that appear to contain essential regulatory regions. Most of the activity that finally leads to the lethal proteolytic breakdown of cellular target proteins is exerted by downstream caspases sensitive to DEVD oligopeptides (caspase-3 and caspase-7).

#### 4. Mechanisms of Caspase Activation

Once downstream caspases that execute the lethal cuts to vital cellular components are activated, cell death appears to be inevitable. Therefore, understanding the mechanisms that initiate proteolytic activation of caspases is a crucial step in defining targets that allow for the modulation of apoptotic cell death. Recent data suggest that activation of caspases may take place either within death receptor complexes of the cytoplasmic membrane or by a mitochondrion-dependent mechanism within the cytosol (Figure 3).

#### Death Receptor Pathway

One of the best characterized pathways for the initiation of apoptosis involves the binding of extracellular death signal proteins (TNF- $\alpha$ , FasL, TRAIL, and Apo-3L) to their cognate cell surface receptors.<sup>59-61,61a</sup> At present, the cDNA sequences of 5 death receptors are known (Table 3).<sup>62-67</sup> The death receptors contain a distinct cytoplasmic domain comprising  $\approx 80$  amino acid residues that is critical for their proapoptotic function.<sup>68,69</sup> Given its importance in the transmission of



**Figure 3.** Schematic diagram of the apoptotic death pathway. Caspases are activated by a receptor- or mitochondrion-dependent mechanism. Subsequent cleavage of multiple cellular target proteins finally results in apoptotic cell death. Antiapoptotic mechanisms are indicated in white boxes. FLIP and IAP indicate FLICE-like inhibitory protein and inhibitor of apoptosis protein, respectively.

proapoptotic signals, this domain was designated “death domain.”

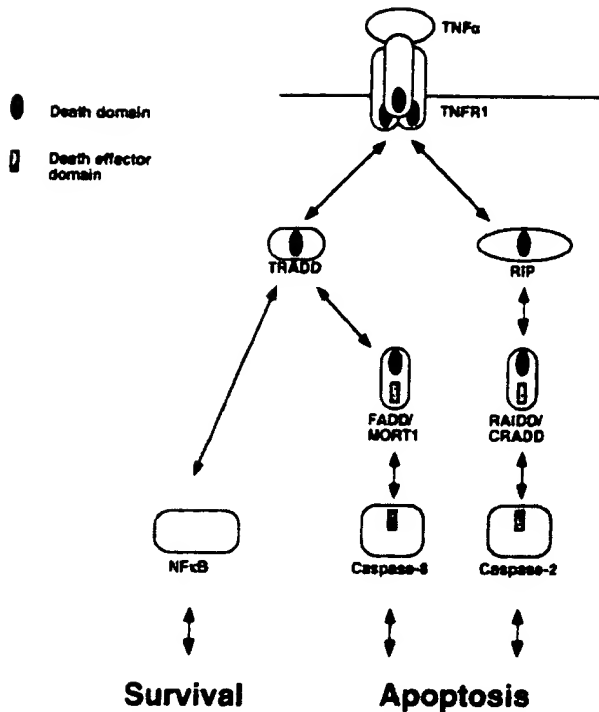
The mRNA for the prototypical death receptor Fas was shown to be detectable in several different organs, including the heart, and in atherosclerotic lesions.<sup>18,70-72</sup> Mice deficient in Fas and mice carrying spontaneous mutations of either FasL (*gld* mice) or Fas (*lpr* mice) exhibit a phenotype of lymphoproliferative and autoimmune disease.<sup>73-75</sup> In patients with Canale-Smith syndrome, a syndrome caused by a mutation of the Fas gene, similar characteristics of lymphoproliferation, autoimmune hemolytic disease, thrombocytopenia, and an additional propensity for neoplasia were described.<sup>76</sup>

Signal transduction through the death receptor involves a unique set of proteins that are not part of other signal transduction pathways (Figure 4). After binding of their cognate ligands, the death receptors form a homotrimeric complex and, by virtue of death domain-mediated protein-protein interactions, recruit intracellular adaptor proteins to the cell membrane. In the case of TNF- $\alpha$  receptor 1 (TNFR1)

**TABLE 3. Death Receptors and Their Ligands**

Receptor	Ligand
Fas (Apo-1CD95)	FasL
TNFR1	TNF- $\alpha$
DR3 (TRAMP, Apo-3, Wsl-1)	Apo-3L
DR4 (TRAIL-R1)	TRAIL
DR5 (TRAIL-R2)	TRAIL

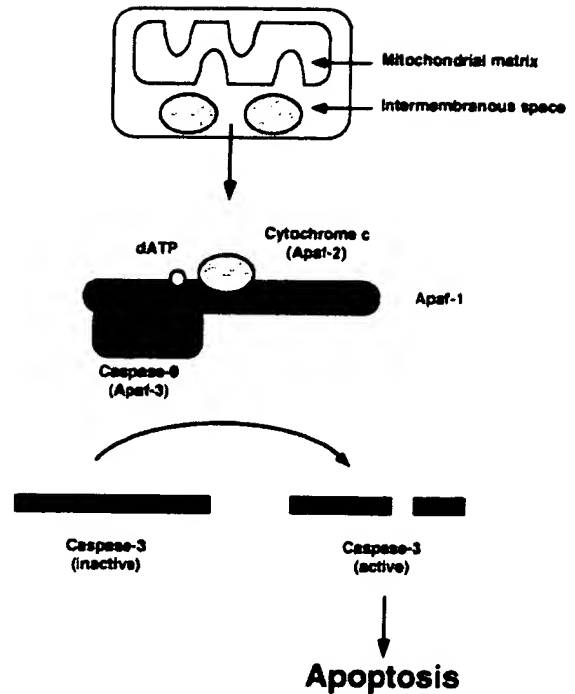
Refer to References 59 to 67.



**Figure 4.** Signaling mechanisms implicated in the transduction of proapoptotic and antiapoptotic signals after stimulation of tumor necrosis factor receptor 1. Protein domains essential for protein-protein interactions and signal transduction are highlighted. CRADD indicates caspase and RIP adaptor with death domain; FADD, Fas-associated death domain protein; FasL, Fas ligand; MORT, mediator of receptor-induced toxicity; NF $\kappa$ B, nuclear factor  $\kappa$ B; RAIDD, RIP-associated ICH-1/Ced-3-homologous death domain protein; RIP, receptor-interaction protein; TNFR, tumor necrosis factor receptor; TRADD, TNFR-associated death domain protein; and TRAIL, TNF-related apoptosis-inducing ligand.

and death receptor 3 (DR3), this is TNFR-associated death domain protein (TRADD), whereas Fas and DR4 interact with Fas-associated death domain protein (FADD).<sup>46,64-66,77,78</sup> FADD and TRADD appear not to interact with DR5, suggesting that additional, so far unknown, proteins may be involved.<sup>67</sup> Signaling induced by activation of TNFR1 or DR3 diverges at the level of TRADD.<sup>78</sup> On the one hand, nuclear translocation of the transcription factor nuclear factor  $\kappa$ B (NF $\kappa$ B) and activation of c-Jun N-terminal kinase (JNK) are initiated.<sup>64,79,80</sup> On the other hand, TNF- $\alpha$  signaling is linked to the Fas signaling pathway through interaction of TRADD with FADD. Surprisingly, FADD knockout mice exhibit a phenotype of ventricular thinning and poorly developed trabeculation of the heart.<sup>80a</sup> An additional death domain-containing protein, receptor-interaction protein (RIP), was also shown to interact with the cytoplasmic domain of TNFR1.<sup>81,82</sup>

Induction of apoptosis by FasL and TNF- $\alpha$  critically depends on caspase activation.<sup>83</sup> FADD was shown to directly interact with caspase-8.<sup>45,46,50</sup> Similarly, caspase-2 can be recruited to RIP through an adaptor protein called RIP-associated ICH-1/Ced-3-homologous death domain protein (RAIDD).<sup>47</sup> Interaction of FADD and RAIDD with the caspases requires the so-called death effector domain (DED)



**Figure 5.** Schematic diagram showing the mechanism of cytochrome c-dependent caspase activation.

that shows homology to the prodomain of the targeted protease.<sup>84</sup>

### Mitochondrial Pathway

Recent reports suggest an important role of the mitochondrion in the induction of apoptotic cell death. Using a cell-free assay, Liu et al<sup>85</sup> defined three proteins in addition to dATP or ATP that are required for caspase-3 activation (Figure 5).<sup>85</sup> Surprisingly, one of the proteins was identified as the mature heme-containing form of cytochrome *c* that is located in the mitochondrial intermembranous space under physiological conditions.<sup>85-88</sup> The release of cytochrome *c* into the cytoplasm appears to be a crucial step for this mechanism of caspase activation. As a potential release mechanism, mechanical rupture of the outer mitochondrial membrane secondary to mitochondrial swelling was suggested.<sup>89</sup> However, it has to be kept in mind that in most apoptotic cells, mitochondria are not obviously swollen.<sup>2</sup>

Interestingly, the second protein, termed apaf-1, is considered to be a mammalian homologue of the proapoptotic factor ced-4 of *C. elegans*.<sup>90</sup> The third protein involved, apaf-3, was recently shown to be identical to caspase-9.<sup>91</sup> Interaction between caspase-9 and apaf-1 is mediated through homologous regions in the prodomain of the caspase and the amino terminus of apaf-1 and depends on the presence of cytochrome *c* and dATP ( $>1 \mu\text{mol/L}$ ) or ATP ( $>1 \text{ mmol/L}$ ). Nonhydrolyzable ATP analogues are nonfunctional, indicating that cytochrome *c*-mediated caspase activation may be an energy-dependent process.<sup>91</sup>

In addition to the release of cytochrome *c*, an alternative mechanism involving the release of another mitochondrial protein, termed apoptosis-inducing factor (AIF), has been proposed.<sup>92</sup> Unlike cytochrome *c*, AIF proved to have a

proteolytic activity that could be blocked by a broad-spectrum caspase inhibitor but not by inhibitors specific for caspase-1 and -7.<sup>93</sup> The release of AIF was shown to depend on the opening of the mitochondrial permeability transition pore that results in the breakdown of the proton and electrical gradients over the inner mitochondrial membrane.<sup>94</sup> In contrast, cytochrome *c* release was independent of the formation of the permeability transition pore and even occurred when the gradients over the inner mitochondrial membranes were maintained.<sup>86,87</sup>

### 5. Regulatory Proteins

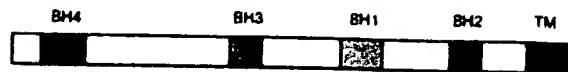
Given the fact that the effector machinery for apoptotic cell death is already in place in all nucleated metazoan "cell types," it is not surprising that mechanisms have evolved that allow for a tight regulation of apoptosis.<sup>95</sup> Recent evidence indicates that regulatory mechanisms affect different levels in the sequence of apoptotic events. In the mammalian cell system, inhibitory proteins have been characterized that prevent the activation of caspases as well as inhibit the proteolytic activity of caspases (Figure 3). Interestingly, most of the strategies for the inhibition of apoptosis are copied by viruses that are capable of maintaining a persistent infection of the host cell.

#### Bcl-2 Protein Family

Genetic analysis in the nematode *C. elegans* defined a genetic locus, *ced-9*, whose loss-of-function mutation caused apoptosis, thus defining a negative regulator of apoptosis.<sup>96</sup> The mammalian homologue *bcl-2* was initially discovered by virtue of its reciprocal translocation (t14;18) to the immunoglobulin heavy chain locus in follicular B-cell lymphomas and in acute lymphoblastic leukemia, promoting survival of neoplastic cells.<sup>97</sup> Expression of human *bcl-2* in *C. elegans* can functionally substitute for the deficiency of *ced-9*, suggesting a high degree of functional conservation during evolution.<sup>98</sup> As in the case of caspases, mammalian cells rely on a whole family of proteins structurally related to *ced-9* (Figure 6). In overexpression experiments, members of the mammalian *bcl-2* protein family were shown to mediate both proapoptotic and antiapoptotic regulation.<sup>99-110</sup>

Functional importance of *bcl-2*-related proteins is suggested by the phenotypic alterations observed in mice deficient in the antiapoptotic regulators *bcl-2* and *bcl-x*. *Bcl-2* knockout mice are characterized by massive apoptotic cell death of lymphocytes in lymphoid tissues as well as by polycystic kidneys, hypopigmented hair, and intestinal abnormalities.<sup>111-114</sup> Interestingly, despite widespread *bcl-2* expression in the fetus, intrauterine development was not impaired, and progressive reduction in body weight and increased mortality became obvious only during the postnatal period. Isolated *bcl-2*<sup>-/-</sup> thymocytes had a markedly increased sensitivity to dexamethasone- and irradiation-induced apoptosis.<sup>113</sup> In contrast to *bcl-2* knockout mice, mice deficient in the antiapoptotic regulator *bcl-x* died in utero at approximately day 13.<sup>115</sup> Histological analysis showed impaired hematopoiesis and deregulated brain development. The phenotypic differences in the knockout studies suggest that the genes for regulatory proteins may not necessarily function interchange-

#### Anti-apoptotic

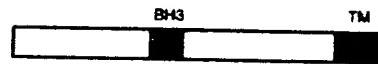


Bcl-2  
Bcl-x<sub>L</sub>  
Mcl-1  
Bcl-w  
A1

#### Pro-apoptotic



Bax  
Bak  
Bad (TM domain missing)



Bik  
Hrk  
Bid (TM domain missing)  
Bcl-x<sub>s</sub> (contains BH4 domain)

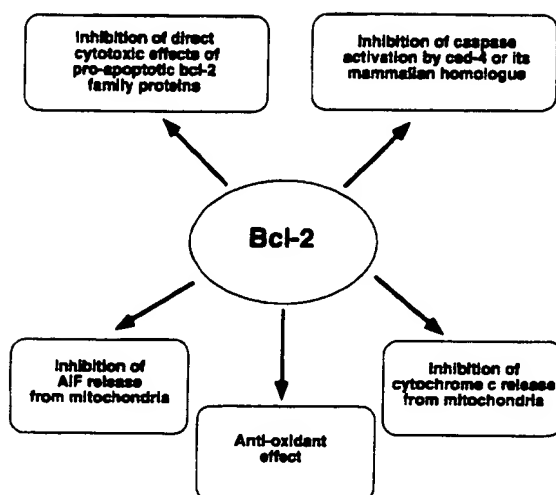
**Figure 6.** Structural features of antiapoptotic and proapoptotic members of the *bcl-2* protein family. BH1 and TM indicate *bcl-2* homology domain and transmembrane domain, respectively.

ably because of either a tissue-specific expression pattern or true functional differences. The targeted disruption of the proapoptotic *bax* gene resulted in a phenotype characterized by lymphoid hyperplasia, hyperplasia of ovarian granulosa cells, and male infertility due to impaired spermatogenesis in the testes.<sup>116</sup>

Interestingly, abnormalities in the cardiovascular system have not been observed in any of these knockout studies. This, however, does not preclude the possibility that proteins of the *bcl-2* protein family are of functional importance in the cardiovascular system, as the loss of one antiapoptotic protein may be compensated for by other members of the same protein family or by alternative antiapoptotic mechanisms. Expression of *bcl-2*, *bcl-x<sub>L</sub>*, and *bax* has been documented in cardiomyocytes and in atherosclerotic lesions by immunohistochemistry.<sup>71,72,117-119</sup> In addition, overexpression of *bcl-2* in cardiomyocytes and vascular smooth muscle cells in vitro can prevent apoptotic cell death induced by p53, indicating that all other proteins required for *bcl-2* to exert its antiapoptotic function are functional in these cells.<sup>120,121</sup>

Structurally, proteins of the *bcl-2* family contain different combinations of 4 conserved domains termed BH1 (*bcl-2* homology), BH2, BH3, and BH4 (Figure 6).<sup>103,122,123</sup> In most *bcl-2*-related proteins, localization to the outer mitochondrial and nuclear membranes as well as to the endoplasmic reticulum is mediated by a carboxy-terminal transmembrane domain.<sup>124,125</sup> All antiapoptotic *bcl-2*-related proteins contain BH1, BH2, BH4, and transmembrane domains. In contrast, the minimal structural requirement for proapoptotic members of this protein family is an intact BH3 domain.<sup>108,109</sup> Surprisingly, the 3-dimensional structure of *bcl-x<sub>L</sub>* showed a close





**Figure 7.** Mechanisms suggested to mediate the antiapoptotic effect of bcl-2. AIF indicates apoptosis-inducing factor.

similarity to bacterial proteins that form transmembrane channels, allowing for the translocation of bacterial peptide toxins.<sup>126</sup> In fact, integration of bcl-x<sub>L</sub> into artificial membranes created a large nonselective transmembrane ion conductance.<sup>127</sup>

Four principal mechanisms for the bcl-2-mediated antiapoptotic effect have been proposed. These include (1) a direct antioxidant effect, (2) inhibition of the release of proapoptotic mitochondrial proteins, (3) sequestration and/or modulation of the proapoptotic ced-4 protein and its mammalian homologue, and (4) inhibition of a direct cytotoxic effect of the proapoptotic regulators bax and bak (Figure 7). The generation of reactive oxygen species was initially suggested to be a common final pathway of apoptosis that could be abrogated by the antioxidant activity of bcl-2.<sup>128</sup> However, later observations indicating that bcl-2 was protective against staurosporine-induced apoptosis even under hypoxic conditions, where the generation of reactive oxygen species is markedly reduced, called this mechanism as the general function of bcl-2 into question.<sup>129</sup>

Increasing evidence suggests that the function of bcl-2 is strongly related to the regulation of protein translocation from the mitochondrial intermembrane space into the cytosolic compartment. Overexpression of bcl-2 effectively blocked the release of cytochrome *c* from mitochondria into the cytosol.<sup>86,87</sup> This finding is in concordance with previous data indicating that activation of caspase-3, -6, and -7 is downstream from bcl-2.<sup>54,130,131</sup> Likewise, bcl-2 blocked the release of AIF, suggesting that bcl-2 and its antiapoptotic congeners may function as a "guardian" against mitochondrial initiation of caspase activation.<sup>93</sup>

An intriguing finding is the observation that ced-9, the *C. elegans* homologue of mammalian bcl-2, directly interacts with ced-4, a proapoptotic protein in *C. elegans* that is involved in the activation of the caspase ced-3.<sup>132,133</sup> Actually, ced-4 is essential for ced-9 to execute its antiapoptotic function.<sup>134</sup> As outlined above, the mammalian ced-4 homologue apaf-1 forms a complex with cytochrome *c*, (d)ATP, and caspase-9 to activate caspase-3. Given the fact that

human bcl-x<sub>L</sub> can interact with ced-4, it seems plausible that antiapoptotic bcl-2 family proteins directly interfere with cytochrome *c*-dependent caspase activation.<sup>91,132</sup> In addition, direct competition of bax with ced-4 to bind to bcl-x<sub>L</sub> was proposed to mediate the proapoptotic effect of bax.<sup>132</sup>

However, overexpression of bax and bak in the yeast *Saccharomyces cerevisiae*, which does not contain endogenous bcl-2 family proteins, was shown to induce cell death, indicating that bax and bak have a direct cytotoxic effect independent from the inactivation of antiapoptotic bcl-2 proteins.<sup>135</sup> Cell death could be blocked by concomitant expression of antiapoptotic bcl-2 family proteins. Part of the antiapoptotic effect of bcl-2 may therefore be attributed to the inhibition of a direct cytotoxic effect of bax and bak.<sup>135</sup>

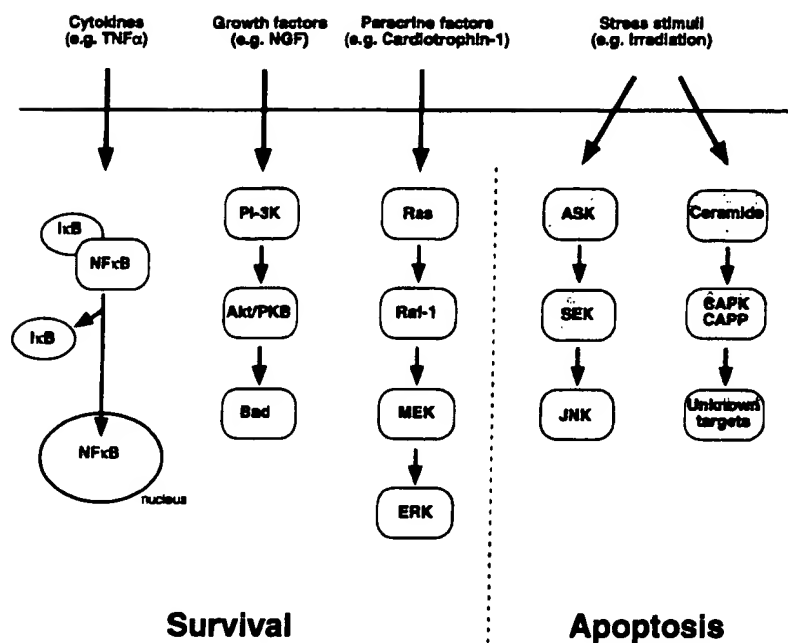
### Inhibitor of Apoptosis Proteins

The prototypical form for this family of proteins was initially isolated from baculovirus, a virus infecting insect cells.<sup>136</sup> In the mammalian system, five homologues have been identified; they are termed X-linked IAP, neuronal IAP, c-IAP1, c-IAP2, and survivin.<sup>137-139</sup> c-IAP1 and c-IAP2 were shown to bind to TNF- $\alpha$  receptor-associated factor 1 and 2 (TRAF1 and TRAF2) and thus can be recruited to the activated TNFR complex.<sup>140</sup> c-IAP2 was suggested to be involved in TNF- $\alpha$ -mediated activation of the NF- $\kappa$ B pathway that confers protection against apoptosis.<sup>141</sup> Direct inhibition of caspase activity was recently shown to be an alternative antiapoptotic mechanism of this class of proteins. X-linked IAP, c-IAP1, and c-IAP2 were shown to interact with and inhibit downstream caspases (caspase-3 and -7), thereby inhibiting apoptosis initiated either by receptor-mediated or by cytochrome *c*-dependent mechanisms.<sup>142,143</sup>

### Inhibition of Receptor-Mediated Caspase Activation

As outlined above, initiation of apoptosis by death receptor ligands requires the recruitment of proteins to the activated death receptors mediated through death domains and DEDs. Database searches led to the isolation of viral proteins that contain DEDs and promote cell survival when overexpressed in cells.<sup>144,145</sup> Mammalian homologues of the viral inhibitors termed FLIP, I-FLICE, CASH, and FLAME-1 show a high degree of homology to caspase-8 and -10, although the protease domain appears to be nonfunctional.<sup>146-149</sup> A similar nonfunctional homologue of caspases-2 and -8 termed apoptosis repressor with caspase recruitment domain (ARC) appears to be selectively expressed in skeletal and cardiac muscle.<sup>149a</sup> Most likely, this class of proteins competitively inhibits receptor-induced activation of upstream caspases. In addition, a TRAIL receptor lacking the intracytoplasmic region essential for transduction of the death signal was isolated.<sup>150</sup> Because this receptor is attached to the cell membrane through a phosphatidylinositol anchor, it can be released enzymatically.<sup>150</sup> The death receptors Fas and TNFR1 were also shown to exist in soluble forms.<sup>151,152</sup> At present, it is not known whether these soluble death receptors exert a physiological role as scavengers for death receptor ligands.





**Figure 8.** Signal transduction pathways implicated in the regulation of cell survival and apoptosis. ASK indicates apoptosis signal-regulating kinase; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatase; ERK, extracellular signal-regulated kinase; I $\kappa$ B, inhibitor of nuclear factor  $\kappa$ B; JNK, c-Jun N-terminal kinase; MEK, MAPK/ERK kinase; NF $\kappa$ B, nuclear factor  $\kappa$ B; NGF, nerve growth factor; PI-3K, phosphatidylinositol-3 kinase; PKB, protein kinase B; SEK, SAPK/ERK kinase; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; TRAF2, TNF- $\alpha$  receptor-associated factor 2.

## 6. Signal Transduction

Only recently have efforts been made to disentangle the intricate relationships between signal transduction and apoptosis (Figure 8). Analysis is complicated by the fact that receptor agonists may activate several signal transduction mechanisms with opposing effects on apoptosis regulation.

Stimulation of TNFR1 and DR3 activates the nuclear transcription factor NF $\kappa$ B, a heterodimeric complex composed of a Rel subunit (RelA, RelB, or c-Rel) and either the p52 or p50 subunit.<sup>153</sup> I $\kappa$ B sequesters and thus inactivates NF $\kappa$ B in the cytoplasm. Degradation of the inhibitory subunit I $\kappa$ B by a ubiquitin-dependent pathway allows for the nuclear translocation of NF $\kappa$ B and transcriptional transactivation of target genes. Using an I $\kappa$ B mutant that resists inactivation, cell viability was markedly reduced after treatment with TNF- $\alpha$ , daunorubicin, or irradiation.<sup>154–156</sup> Furthermore, mouse fibroblasts deficient in the RelA subunit of NF $\kappa$ B became sensitive to TNF- $\alpha$ , whereas the parental cell line was resistant to the proapoptotic effect of TNF- $\alpha$ , indicating that the apoptotic activity of TNF- $\alpha$  depends on whether NF $\kappa$ B signaling is inactivated in addition to the recruitment of FADD and caspase-8.<sup>157</sup> In fact, RelA knockout mice die in utero because of liver atrophy due to massive apoptosis of hepatocytes.<sup>158</sup>

Survival factors like interleukin-3, nerve growth factor (NGF), insulin-like growth factor-1 (IGF-1), or platelet-derived growth factor protect cells from undergoing apoptotic cell death. The cognate receptors belong to the family of protein tyrosine kinase receptors that are implicated in the activation of phosphatidylinositol-3 kinase (PI-3 kinase). Rescue of PC12 pheochromocytoma cells with NGF or IGF-1 depends on the activation of PI-3 kinase.<sup>159–161</sup> Downstream from PI-3 kinase, Akt (also known as protein kinase B [PKB]) was found to be critical for the prevention of apoptotic cell death.<sup>161–163</sup>

The discovery that bad, a proapoptotic bcl-2 family protein, can be phosphorylated by PKB/Akt provided the first direct link between a growth factor signal transduction pathway and apoptosis regulatory proteins.<sup>164</sup> Phosphorylated bad was shown to bind less efficiently to membrane-associated bcl-x<sub>L</sub>.<sup>165</sup> Because bad lacks a carboxy-terminal membrane anchor, reduced affinity for membrane-associated bcl-x<sub>L</sub> results in the translocation of bad from the intracellular membrane fraction to the cytosol, where it is sequestered in a complex with 14-3-3 proteins. In accordance with this, substitution of two specific serine phosphorylation sites increases the proapoptotic activity of bad.<sup>165</sup>

In mammalian cells, four parallel kinase cascades have been described that finally lead to the activation of members of the mitogen-activated protein kinase (MAPK) family, such as the ERKs (p42 and p44), JNK (alternatively called SAPK), and p38 protein kinase.<sup>166,167</sup> In several cell types, a proapoptotic role for the kinase cascade MEKK1-SEK-JNK was shown.<sup>168–172</sup> A similar role has been suggested for the p38 kinase cascade, although available evidence is limited.<sup>168,173</sup> Activation of the JNK pathway is implicated in the initiation of apoptosis by different stress stimuli. In a recent report, even Fas-induced apoptosis was shown to be partly dependent on JNK activation, which was mediated by a novel signal transduction molecule termed Daxx.<sup>174</sup> Likewise, apoptosis signal-regulating kinase 1 (ASK1), an upstream activator of the JNK kinase (SEK), was shown to promote TNF- $\alpha$ -induced apoptosis.<sup>175</sup> However, in SEK knockout mice, apoptosis triggered by several stress stimuli was not impaired in thymocytes compared with control cells.<sup>176</sup> Further study is required to elucidate the proapoptotic pathways that involve JNK and p38.

Increasing evidence suggests that activation of the Ras-Raf-1-MEK-ERK pathway is protective against apoptotic cell death. Raf-1 is targeted by bcl-2 to the outer mitochondrial membrane, where it phosphorylates bad.<sup>177</sup> However, the

functional significance of this is not clear, since phosphorylation does not involve the serine residues essential for the cytoplasmic sequestration of bad.<sup>165</sup> Constitutively active MEK rescues neuronal cells from apoptotic cell death induced by growth factor withdrawal.<sup>160,168</sup> Cardiomyocytes were shown to be protected from apoptotic cell death after serum deprivation by cardiotrophin-1.<sup>178</sup> Interestingly, the protective effect of cardiotrophin-1 could be blocked by an inhibitor of MEK. Furthermore, blockade of ERK activation augmented apoptosis induced by oxidant stress in neonatal rat ventricular cardiomyocytes, suggesting that the ERK pathway can mediate antiapoptotic signaling in neonatal cardiomyocytes.<sup>179</sup> The antiapoptotic effect of bcl-2 depends on serine phosphorylation between the BH3 and BH4 domains.<sup>180</sup> Signaling through the Raf-1-MEK-ERK pathway has been implicated in the phosphorylation of bcl-2, providing a potential link between ERK activation and cell survival.<sup>180,181</sup>

An additional signaling mechanism related to the regulation of apoptosis involves the formation of ceramide from sphingomyelin.<sup>169,182</sup> Interestingly, generation of ceramide appears to be an early event in some forms of stress-induced apoptosis, being detectable within only 10 minutes. Cells from patients with Niemann-Pick disease (hereditary deficiency of acid sphingomyelinase) and from knockout mice deficient in acid sphingomyelinase exhibited a markedly decreased sensitivity to radiation-induced apoptosis.<sup>183</sup> Although several downstream targets in ceramide signaling have been recognized, including protein kinase C  $\zeta$  (PKC $\zeta$ ), ceramide-activated protein phosphatase (CAPP), and ceramide-activated protein kinase (CAPK), the mechanisms that link ceramide signaling pathways to the activation of caspases are still incompletely understood.<sup>182</sup>

### 7. p53 and Apoptosis

Several reports have established a relationship between DNA damage, cell cycle control, and apoptosis. Inducers of apoptosis that cause DNA damage (eg, UV- or  $\gamma$ -irradiation and chemotherapeutic drugs) proved to depend on functional p53.<sup>184,185</sup> In addition, when cells are induced to proliferate by deregulated expression of the adenoviral oncoprotein E1A or the oncogene *c-myc*, they undergo apoptosis, unless they are rescued by bcl-2 or its adenoviral homologue E1B.<sup>186-188</sup> In serum-starved fibroblasts, apoptosis was clearly related to crossing the G<sub>1</sub>/S cell cycle checkpoint, as only those cells that showed evidence for DNA synthesis became apoptotic.<sup>189</sup> Interestingly, cell cycle reentry, DNA synthesis, and apoptotic cell death have also been observed in neonatal and cardiac cardiomyocytes overexpressing the positive cell cycle regulators E2F-1 or adenoviral E1A.<sup>190-192</sup> Apoptosis induced by forced cell cycle reentry was reported to depend on p53 in fibroblasts and kidney cells.<sup>193-195</sup> However, despite the capacity of neonatal cardiomyocytes to undergo apoptosis in response to p53 overexpression, myocyte apoptosis induced by forced entry into the S phase of the cell cycle was shown to occur in p53-deficient mice.<sup>120,192,196</sup>

The proapoptotic effect of p53 has been linked to the p53-induced expression of Fas, bax, and IGF binding protein-3.<sup>197-200</sup> In an extensive analysis of the gene expression pattern during p53-mediated apoptosis of a colon cancer cell line, 7

of 14 genes that were induced at least 10-fold by p53 were involved in cellular redox reactions.<sup>201</sup> Since antioxidants reduced the extent of p53-induced apoptosis, oxidative stress seems to constitute an important intermediary step in p53-mediated apoptosis.<sup>201</sup>

### 8. Evidence for Apoptosis in the Cardiovascular System

Although apoptosis has long been recognized as a principal mechanism for the elimination of redundant, autoreactive, or neoplastic cells, only recently a critical role of apoptosis was suggested in several cardiovascular diseases (Table 4).

#### Apoptosis in Cardiac Development

During cardiac development, programmed cell death was suggested to be of importance in the formation of septal, valvular, and vascular structures, implicating the potential importance of either excessive or inappropriate apoptosis in congenital heart disease.<sup>202</sup> However, so far, direct evidence for an apoptotic cell death by TUNEL staining has been provided only for mesenchymal cells in the bulbus cordis of the rat heart at 14 and 16 days of gestation.<sup>203</sup> Excessive apoptosis of the cardiac conduction system was suggested to be a possible mechanism in the pathogenesis of heart block.<sup>204,205</sup> On the other hand, incomplete apoptotic cell deletion has been postulated to cause the persistence of accessory atrioventricular conduction pathways, such as in Wolff-Parkinson-White syndrome.<sup>204</sup>

#### Apoptosis and Heart Failure

The factors that lead to the development and progression of heart failure are still not fully understood. Besides myocyte hypertrophy, myocyte dysfunction due to altered calcium homeostasis, impaired myofilament Ca<sup>2+</sup> sensitivity, fiber slippage, and myocardial fibrosis, progressive loss of cardiomyocytes is considered to play a major contributory role.<sup>206-208</sup> In canine models of pacing-induced heart failure and heart failure due to chronic ischemic injury, loss of cardiomyocytes due to apoptosis was detectable by TUNEL staining, whereas in control myocardium only rare cardiomyocytes stained positive.<sup>13,209</sup> Narula et al<sup>210</sup> reported that in myocardial specimens from patients undergoing cardiac transplantation, apoptosis detected by TUNEL staining was consistently observed in idiopathic dilated cardiomyopathy but not in ischemic cardiomyopathy. However, in a more recent study, this difference with regard to the etiology of heart failure

TABLE 4. Cardiovascular Diseases Associated With Apoptosis

Disease	Reference
Dilated cardiomyopathy	118, 210
Ischemic cardiomyopathy	118
Arrhythmogenic right ventricular dysplasia	211
Acute myocardial infarction	216-219
Atherosclerosis	15-17, 72, 228, 229
Myocarditis	250, 251
Cardiac allograft rejection	245
Preexcitation syndromes	204
Congenital atrioventricular block	204, 205

could not be confirmed.<sup>118</sup> Furthermore, in a series of patients with arrhythmogenic right ventricular dysplasia (a myocardial disease characterized by fibrofatty replacement of right ventricular cardiomyocytes and a high incidence of ventricular arrhythmia and sudden cardiac death), histological evidence for myocyte loss due to apoptotic cell death has been shown in 6 of 8 patients.<sup>211</sup> Notably, an infiltration by inflammatory cells was absent in the tissue sections of patients with heart failure, indicating that cell-mediated cytotoxicity by immune cells appears not to play a major role under these conditions. In heart failure, the apoptotic index, ie, the number of TUNEL-positive nuclei per 100 nuclei, was reported to be as high as 35.5% in the initial study.<sup>210</sup> Given the fact that the TUNEL-positive state after apoptotic cell death may last <24 hours, this high degree of myocyte loss would lead to rapid organ destruction. Much lower values for the apoptotic index (0.2% to 0.4%) that are still >100-fold above control values may therefore more reliably reflect the overall extent of ongoing myocyte apoptosis in heart failure.<sup>13,118</sup>

Potential mechanisms for the induction of apoptotic cell death at the cellular level may involve mechanical factors or elevated levels of neurohumoral factors. In an experimental model of isometric stretch of papillary muscle, apoptosis of cardiomyocytes could be detected in 0.64% of cardiomyocytes by TUNEL staining, indicating that volume overload and elevated end-diastolic left ventricular pressure may constitute an initiating event for myocyte apoptosis.<sup>212</sup> After aortic banding in rats, apoptosis of myocytes could be verified in tissue sections, further emphasizing the potential role of hemodynamic factors. Peak cell loss was observed 4 days after aortic banding.<sup>213</sup>

Kajstura et al<sup>214</sup> observed an increased percentage of apoptotic cells in isolated adult cardiomyocytes after treatment with angiotensin II (0.9% apoptotic cells in angiotensin II-treated cells versus 0.2% in control cells). This effect was mediated by AT<sub>1</sub> angiotensin II receptors, raising the possibility that the beneficial effect of angiotensin-converting enzyme inhibitors or AT<sub>1</sub> receptor blockers in heart failure may in part be attributable to an inhibition of myocyte loss. Recently, atrial natriuretic factor (ANF) was shown to increase the apoptotic index from 4.8% to 19% in isolated neonatal cardiomyocytes.<sup>215</sup> Because ANF levels are elevated in heart failure, sensitivity of cardiomyocytes to ANF may be of pathophysiological importance. In addition, reexpression of the ANF gene in hypertrophied ventricular cardiomyocytes may expose these cells to markedly increased local levels of ANF and thus may promote transition from myocardial hypertrophy to heart failure by inducing apoptotic cell loss. However, it is somewhat counterintuitive to assume that ANF is produced at high levels in the atrial and fetal ventricular myocytes, and there is no evidence of apoptosis so far reported in these cells *in vivo*.

### Apoptosis in Ischemic Heart Disease

Although myocardial infarction was long considered to be characterized by nonapoptotic ("necrotic") cell death due to the breakdown of cellular energy metabolism, there is growing evidence that myocyte loss during the acute stage of myocardial infarction involves both apoptotic and nonapo-

ptotic cell death.<sup>216-218</sup> In human postmortem studies of myocardial infarction, apoptotic cardiomyocytes appeared to be predominantly localized in the hypoperfused border zone between the central infarct area and noncompromised myocardial tissue.<sup>218,219</sup> Interestingly, myocytes in the peri-infarct region were shown to upregulate the apoptotic regulatory proteins bax and bcl-2.<sup>71,117</sup> In addition, myocytes showing evidence of DNA degradation, chromatin condensation, and cell fragmentation were detected in human hibernating myocardium.<sup>14</sup> Observation in animal models of myocardial infarction suggest that apoptosis may contribute substantially to cell death even within the central infarct area with 5% to 33% of the cardiomyocytes staining positive for DNA fragmentation.<sup>71,220-222</sup> However, at present, the relative importance of apoptotic and nonapoptotic cell death in both the acute and chronic phases of myocardial infarction is not known. Initial evidence for the potential pathophysiological significance of apoptosis has recently been provided in a rat model of myocardial infarction.<sup>222</sup> Treatment with the caspase inhibitor zVAD.fmk led to a reduction in infarct size and an improvement of acute functional parameters. However, these measurements were obtained 24 hours after infarction, and it is not known whether the beneficial effects of zVAD.fmk persists in the chronic stage.

Ischemia is associated with multiple alterations in the extracellular and intracellular milieu of cardiomyocytes that may act as inducers of apoptosis. So far, a proapoptotic role has only been verified in *in vitro* experiments for hypoxia, whereas the role of acidosis or elevated adenosine concentrations is not known.<sup>196,223</sup> As hypoxia increases transcriptional transactivation by p53 in neonatal rat ventricular cardiomyocytes, a p53-mediated mechanism for myocyte apoptosis under hypoxic conditions was suggested.<sup>120,196</sup> However, the extent of apoptosis in hypoxic regions after ligation of the left coronary artery in p53-deficient mice was shown to be similar to that in wild-type mice.<sup>220</sup> The death receptor Fas is markedly upregulated in cardiomyocytes during ischemia and hypoxia, and cardiomyocytes may thus become susceptible to apoptotic cell death by interaction with FasL. Whereas under control conditions <1% of cardiomyocytes expressed the Fas antigen, Fas was detectable in >50% of cardiomyocytes within a few hours of ischemia and ischemia/reperfusion.<sup>71,224</sup> It is not known, however, whether FasL, which is essential to trigger apoptotic cell death by a Fas-dependent mechanism, is expressed in the ischemic myocardium.

The role of apoptosis in reperfusion injury has recently been addressed in rat and rabbit animal models, where reperfusion was shown to accelerate the occurrence of apoptotic cell death in cardiomyocytes.<sup>221,225</sup> Because the formation of reactive oxygen species has been implicated as one of the pathomechanisms for tissue injury during reperfusion, the recent finding that oxidative stress induces apoptosis in isolated neonatal rat ventricular cardiomyocytes may provide an important mechanistic link between reperfusion and tissue injury.<sup>179,226</sup>

In addition, apoptotic cell death may have a role in the remodeling of noninfarcted myocardium, as evidenced in human myocardial specimens sampled within 10 days after

myocardial infarction.<sup>219</sup> In myocardium remote from the infarcted area, 0.7% of the cardiomyocytes were apoptotic, whereas in control hearts no myocyte apoptosis was detectable. Interestingly, apoptosis in noninfarcted regions of myocardium was inhibited by overexpression of IGF-1 in a transgenic mouse model, resulting in reduced ventricular dilation and wall stress 7 days after infarction.<sup>227</sup> IGF-1 has been shown to activate the antiapoptotic kinase Akt in PC12 cells, but it is not known whether the same mechanism is operative in cardiac myocytes.

### Apoptosis and Atherosclerosis

Apoptosis may prove to play an essential role in atherosclerotic alterations of the vessel wall. In human specimens from atherosclerotic lesions of native coronary vessels and saphenous vein grafts, widespread apoptosis was detectable by TUNEL staining (up to 43% of cells in the lipid-rich core of atheromata).<sup>15-17,72,228,229</sup> Apoptotic cells were often arranged in cell clusters and primarily consisted of macrophages and smooth muscle cells. A substantial number of cells undergoing apoptosis were immunoreactive with a polyclonal antiserum directed against caspase-1 and -3.<sup>228,230</sup> Remarkably, apoptosis did not occur in medial smooth muscle cells.<sup>16</sup> A similar spatial restriction of smooth muscle cell apoptosis to the superficial neointima was observed after denudation of the rat aorta.<sup>231</sup> In that model, apoptosis was no longer detectable after reendothelialization.

In contrast to primary atherosclerotic lesions, where apoptosis was not a consistent finding in all specimens, almost all atherectomy specimens from restenotic lesions showed evidence of apoptosis.<sup>229</sup> Apoptosis strongly correlated with the presence of intimal hyperplasia.<sup>229</sup> In a rat model of balloon vascular injury, apoptosis primarily affected neointimal smooth muscle cells 7 to 28 days after dilation.<sup>16</sup> In contrast, Perlman et al<sup>232</sup> found extensive apoptosis of medial smooth muscle cells with 70% TUNEL-positive cells as early as 0.5 to 2 hours after balloon injury in rat carotid and rabbit iliac arteries. The difference in time course between neointimal and medial smooth muscle cell apoptosis suggests that balloon vascular injury may directly induce apoptosis in medial smooth muscle cells, whereas apoptosis of neointimal smooth muscle cells may be associated with the restructuring of the neointima.

Vascular smooth muscle cells undergo p53-dependent apoptosis after overexpression of the positive cell cycle regulators *c-myc* or E1A.<sup>121,188</sup> Interestingly, isolated vascular smooth muscle cells from human atherosclerotic plaques were shown to have a higher propensity for both spontaneous apoptosis and apoptosis induced by overexpression of p53 compared with vascular smooth muscle cells from normal vessels.<sup>233,234</sup>

An alternative mechanism may involve the induction of apoptosis by a death receptor-dependent mechanism. Fas is known to be widely expressed in human atherosclerotic lesions, including a sizable fraction of smooth muscle cells.<sup>18,72</sup> Twenty percent of Fas-positive cells showed evidence for internucleosomal DNA fragmentation with associated morphological features of apoptosis, like chromatin condensation and nuclear fragmentation. Interestingly, cultured aortic smooth muscle cells were not sensitive to a

cytotoxic anti-Fas antibody despite Fas expression on one third of the cells.<sup>18</sup> However, pretreatment with  $\gamma$ -interferon, interleukin-1, and TNF- $\alpha$  markedly sensitized smooth muscle cells to Fas-induced apoptosis. These cytokines increased both the fraction of Fas-expressing cells to  $\approx 90\%$  of all cells and the density of Fas antigen on individual cells. Interestingly, the combination of  $\gamma$ -interferon, interleukin-1, and TNF- $\alpha$  alone already exerted a proapoptotic effect on cultured smooth muscle cells that may involve both NO-dependent and -independent mechanisms.<sup>228,235</sup> These observations underline the importance of inflammatory cytokines generated by activated infiltrating T lymphocytes ( $\gamma$ -interferon) and macrophages (interleukin-1 and TNF- $\alpha$ ).<sup>236</sup> Activation of immune cells may involve oxidized low-density lipoprotein (LDL) particles, the cell surface receptor CD40, and its cognate ligand.<sup>237,238</sup> One major clinical implication of apoptotic cell death in atherosclerotic lesions may be a reduced plaque stability. In addition to proteolysis, loss of smooth muscle cells in the fibrous cap of atherosclerotic lesions is known to predispose the lesions to plaque instability and therefore may increase the risk of unstable angina pectoris and acute myocardial infarction.<sup>239</sup> In this respect, it is noteworthy that the death receptor Fas is expressed on as many as two thirds of the cells in the fibrous cap in human atherosclerotic lesions.<sup>18</sup>

In recent studies,<sup>240,241</sup> a potential role of oxidative mechanisms has been suggested in the apoptosis of vascular cells. Cultured endothelial cells undergo apoptosis in response to oxidized LDL, indicating a potential role for apoptosis in the early phases of atherogenesis.<sup>240,241</sup> Sensitivity to oxidized LDL could be reduced by nitric oxide or by calcium channel blockers.<sup>241,242</sup> In addition, apoptosis of vascular smooth muscle may at least partly be attributable to oxidant damage by hydrogen peroxide.<sup>243</sup>

It is interesting to note that exposure of phosphatidylserine on the surface of apoptotic cells can promote thrombin generation *in vitro*.<sup>244</sup> At present, it is not known whether this mechanism contributes to the thrombogenicity of atherosclerotic lesions *in vivo*.

### Other Cardiovascular Diseases

Apoptosis due to immune mechanisms may be of major importance in myocarditis and cardiac allograft rejection. Indeed, in a rat model of heterotopic heart transplantation, Szabolcs et al<sup>245</sup> found extensive apoptosis of cardiomyocytes, endothelial cells, and infiltrating leukocytes. Infiltrating cells consisted initially of lymphocytes, whereas macrophages predominated in later stages, when apoptosis was prominent. It is not known to which extent apoptosis is induced by cytotoxic T lymphocytes through Fas-dependent or granzyme B-dependent mechanisms.

Cardiomyocytes express a functional TNFR1 and can undergo apoptosis after stimulation with TNF- $\alpha$  *in vitro*.<sup>35,246,247</sup> Interestingly, TNF- $\alpha$  was shown to be produced in myocardium, although the cell type was not clearly defined.<sup>248</sup> When TNF- $\alpha$  was highly overexpressed under the control of a strong cardiomyocyte-specific promoter, a phenotype of dilated cardiomyopathy was induced in transgenic mice.<sup>249</sup> Likewise, in an animal model of myocarditis, TNF- $\alpha$

was shown to exert a major role in the pathogenesis of myocardial inflammation, although it is not clear in how far TNF- $\alpha$ -mediated apoptosis contributed to myocardial damage.<sup>250,251</sup>

### 9. Future Directions

Basic research in apoptosis has made a tremendous progress within the past few years and will undoubtedly provide exciting new insights in the near future. Accumulating evidence that apoptosis may be an important feature of several cardiovascular diseases will certainly increase the interest in apoptosis in cardiovascular research. In this respect, several points appear to deserve further investigation. First, although apoptosis has been verified histologically in heart failure, acute myocardial infarction, and atherosclerosis, the role of apoptosis in the pathogenesis of these conditions requires substantiation. It is important to determine whether apoptosis is one of the early causes rather than a terminal event that is associated with the end stage of these disease entities. Second, the true incidence of apoptosis is not clear, with reported values ranging from 0.2% to 35% in heart failure. Concerns about the specificity of TUNEL staining have been raised. In addition, TUNEL staining and morphometry are laborious, and the duration of apoptotic cells being detectable by TUNEL may last only a few hours. DNA laddering, though specific, is not quantitative and sensitive in tissue samples, where a small number of cells (<1%) are undergoing apoptosis. Therefore, we need to improve or develop detection techniques that allow for accurate quantitative detection of apoptosis in cardiovascular tissue. Third, to date, the initiating stimuli of apoptosis in myocardial and vascular cells at the cellular level are not well understood. Recognition of the inducing mechanisms could open up ways to inhibit cell death in cardiovascular tissues and possibly help to define targets for future drug design. Fourth, although end-stage events of apoptosis, such as the activation of downstream caspases (caspase-3, -6, and -7) are likely to be essentially uniform in all cell types, some regulatory mechanisms may be unique to cells in cardiovascular tissues. Elucidation of proapoptotic and antiapoptotic mechanisms in cardiomyocytes and vascular smooth muscle cells could delineate potential targets for intervention. Fifth, although pharmacological caspase inhibition prevents myocyte apoptosis induced by ischemia and reperfusion in short-term experiments,<sup>222</sup> the ultimate fate of the cells is not clear. It is not known whether ischemic myocytes that have initiated the apoptosis pathway and are acutely rescued by caspase inhibition will eventually survive or whether the drug simply delays cell death. Sixth, with respect to the clinical situation, the role of apoptosis as a prognostic marker deserves further study. For example, it remains to be determined whether the degree of apoptosis could provide additional prognostic information in patients with impaired left ventricular function and thus help in therapeutic decision making. Seventh, attempts are being made to induce myocyte proliferation as a potential therapeutic approach for heart failure. However, cardiomyocyte apoptosis in response to forced cell cycle reentry may limit the feasibility of this approach. Clarification of the apoptotic mechanisms involved will therefore be

of major importance. Finally, initial attempts to reduce neointima formation by inducing apoptosis through adenoviral gene transfer have been promising in animal models of balloon vascular injury.<sup>252,253</sup> Likewise, induced apoptosis of smooth muscle cells was associated with a regression of both neointimal and primary atheromatous lesions in rabbits.<sup>119</sup> However, further studies are required to evaluate the potential clinical benefit of this approach, as apoptosis of vascular smooth muscle cells may reduce plaque stability and thus initiate acute coronary events.<sup>239</sup>

Taken together, apoptosis increasingly penetrates the field of cardiovascular research. Several exciting hypotheses need to be tested to determine whether the opportunities offered by the modulation of apoptotic cell death will finally translate into new treatment approaches for cardiovascular disease.

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